In-Gel Trypsin Enzymatic Digestion

Guidelines to protect your samples from contamination with keratin

1. Try to avoid any contact of samples and solutions with dust, skin or hair

2. Clean your bench

3. Wear gloves at all times

4. All reagents should be prepared fresh or aliquots could be used if stored at -20°C (the stock solution validity is 6 months if the validity of the reagent itself is not lower)

5. Use ultra-pure water for all solutions (MilliQ water)

Guidelines for sample submission

1. Provide 10ul of samples in recovery vials* or vials with insert for small volumes for LC-MS/MS analysis

***Autosampler vials appropriate for analysis**

Waters Total Recovery (part number: 186000385C)

Figure 1: Waters Total Recovery Vial

2. Provide samples in 1.5 ml eppendorf tube for MALDI-TOF/TOF analysis.

3. Label your tube with the sample ID.

3. Fill in online sample submission form to provide us with more information about your sample

Solutions of reagents

100% Acetonitrile (CH_3CN, HPLC or LC-MS grade)

50% Acetonitrile

-Dilute a volume of 100% ACN 1:1 in MilliQ water

100 mM ammonium bicarbonate (NH_4HCO_3, MW 79.06)

-0.79 g NH_4HCO_3 in 100 ml MilliQ water

-Store at -20°C in aliquots of 10ml

50mM acetic acid

-Prepare 50 mM acetic acid solution by adding 287.36 µL of glacial acetic acid to 100 mL of ultrapure water.

50mM ammonium bicarbonate

-Dilute 100 mM NH_4HCO_3 stock solution 1:1 using MilliQ water

1M DTT (Dithiothreitol, $HSCH_2(CHOH)_2CH_2SH$, MW 154.24)

- Dissolve 0.77 g DTT in 5 ml MilliQ water

- Store at -20°C in aliquots of 500 µl

85mM DTT (To reduce the proteins: *in-gel* reduction is recommended even if the proteins were reduced prior to an electrophoresis run)

-Dilute 1M IAA stock solution using 50mM ammonium bicarbonate

110 mM IAA (Iodoacetamide, C_2H_4INO, MW184.96)

-Dissolve 56 mg of IAA in 3327 µl of MilliQ water

- Store at -20°C in aliquots of 250 µl

55 mM IAA (To prevent the re-formation of disulphide bridges)

-Dilute 110mM IAA stock 1:1 using 50mM ammonium bicarbonate

20 µg/µl of Trypsin- Pierce Trypsin Protease, MS Grade

(Other enzymes with the same pH tolerance as trypsin can be substituted without modifying conditions. These enzymes includes Chymotrypsin, Asp-N, Glu-C and Lys-C)

- Reconstitute lyophilized trypsin using 50mM acetic acid to 1mg/mL (i.e., add 20µL of 50mM acetic acid to 20µg of lyophilized trypsin).

Dilute 1mg/mL trypsin stock solution to 0.01mg/mL using 50mM ammonium bicarbonate.

IMP: always work with the trypsin in an ice bucket to prevent auto-proteolysis

Procedure

Excision of protein bands from polyacrylamide gels

1. Wash the gel slab with water (2 changes, 10 min each)

2. To excise the bands/spots from the gel use clean nitrile gloves and scalpel

3. Cut as close to the protein band as possible to reduce the amount of background gel

4. Excise a gel piece of roughly the same size from a non-protein containing region of the gel for use as a control

5. Cut the gel pieces into roughly $1mm^3$ cubes

6. Put the gel pieces in clean 0.5 or 1.5 ml eppendorfs

De-staining gel pieces from *Coomassie stained bands/spot

1. Wash the gel pieces with water (15 min shaking)

2. Add 100 µl of 100mM 100 mM ammonium bicarbonate (10 min shaking)

3. Remove buffer with pipette and add 50 µl of 1:1 50 mM ammonium bicarbonate / ACN to gel pieces (10 min shaking)

4. If gel pieces are still blue, repeat steps 2 and 3

5. Remove all liquid and add 100% ACN, enough to cover the gel pieces (30 min at 37°C shaking)

6. After the gel pieces have shrunk (they become white and stick together) remove the acetonitrile

IMPORTANT: Solvent volumes used in the washing steps should roughly equal 5 times the gel volume

De-staining gel pieces from *Silver stained bands/spot

1. Add 200ul of 1:1 potassium ferricyanide/sodium thiosulfate and agitate 20 min in the dark at RT

2. Remove all the liquid

3. Add 200µl of MilliQ water and agitate 20 min at RT

4. Remove all the liquid

5. Repeat this procedure until the bands/spots are transparent

6. Add 30µl of MilliQ water and agitate for 20 min at RT

7. Remove all the liquid

8. Add 30µl of 100% of ACN and agitate for 30 min at 37°C

9. Remove all liquid

IMPORTANT: Solvent volumes used in the washing steps should roughly equal 5 times the gel volume

Reduction and alkylation

1. Swell gel pieces in 30 µl of 10mM DTT in 100mM NH_4HCO_3

2. Reduce for 45 min at 56°C with agitation

3. Remove excess liquid

4. Add 30 µl of 55mM of iodoacetamide in 100mM NH_4HCO_3

5. Alkylate in the dark for 30 min at room temperature with agitation

6. Remove all the liquid

7. Wash with 100 µl of 100mM NH_4HCO_3 for 5 min at room temperature and agitation

8. Remove all the liquid

9. Wash with 100 µl of 100%ACN for 15 min at room temperature and agitation

10. Remove all the liquid

11. Remove ACN and allow gel pieces to air dry for 5-10 minutes.

Trypsin digestion

1. Rehydrate the gel pieces with 50µL of 0.01mg/mL trypsin solution to the sample for 30 min at 4°C.

2. After 15 min, add more digestion buffer if the initial volume has been absorbed by the gel pieces

3. Incubate overnight (16-24 hours) at 37°C

4. After overnight incubation, recover the digest to a new tube and add formic acid to each sample to a final formic acid concentration of 5.0% in order to stop the enzymatic reaction.

Extraction of peptides from the gel (normally used to extract big peptides that don't leak from the gel easily)

1. Add 100 µl of 1% formic acid and incubate for 15 min at RT with shaking, then transfer to the vial containing supernatant

2. Add the same volume of 100%ACN and incubate for 15 min at RT with shaking, pipette off and save the supernatant

3. Add 100µl of 1:1 ACN/Water, leave for 5 min, then transfer to the vial containing supernatant.

4. Add 100µl of 1% formic acid in ACN, leave for 5 min, then transfer to the vial containing supernatant

5. **Dry the digested sample to completion** using the SpeedVac

6. **Resolubilize** the sample peptides:

    6.1 For **MALDI-TOF/TOF** analysis re-dissolve in 10-20 µl of 0.1% Trifluoroacetic Acid (TFA) and use Zip Tip to clean up the sample (please see separate protocol)

6.2 For **LC-MS/MS** analysis re-dissolve in 10-20 µl of 0.1% of formic acid and use Zip Tip to clean up the sample (please see separate protocol)